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Role of Protein Kinase Cα in Signaling from the Histamine H_1 Receptor to the Nucleus

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ABSTRACT

Stimulation of histamine H_1 receptors produced a marked activation of inositol phospholipid hydrolysis, intracellular calcium mobilization, and stimulation of the c-fos promoter in CHO-H1 cells expressing the H_1 receptor at a level of 3 pmol/mg protein. The latter response was determined using a luciferase-based reporter gene (pGL3). This response to histamine was not sensitive to inhibition by pertussis toxin but could be completely attenuated by the protein kinase C (PKC) inhibitor Ro-31-8220, or by 24-h pretreatment with the phorbol esters phorbol 12,13-dibutyrate or phorbol-12-myristate-13-acetate. Several isoforms of PKC can be detected in CHO-H1 cells (α, δ, ϵ, μ, ι, ζ) but only PKCα and PKCδ were down-regulated by prolonged treatment with phorbol esters. Of the two isoforms that were down-regulated, only protein kinase Cα was translocated to CHO-H1 cell membranes after stimulation with either histamine or phorbol esters. The PKC inhibitor Gö 6976, which inhibits PKCα but not PKCδ, was also able to significantly attenuate the c-fos-luciferase response to histamine. The mitogen-activated protein kinase kinase inhibitor PD 98059 markedly inhibited the response to histamine, suggesting that the likely major target for PKCα was the mitogen-activated protein kinase pathway. These data suggest that the histamine H_1 receptor can signal to the nucleus via PKCα after activation of phospholipase Cβ.

The histamine H_1 receptor is a seven-transmembrane spanning receptor that produces its intracellular effects via the activation of the heterotrimeric $G_{Q/11}$ family of G proteins (Hill et al., 1997). Activation of this receptor leads to stimulation of phospholipase Cβ, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to form inositol-1,4,5-trisphosphate and diacylglycerol. Inositol-1,4,5-trisphosphate released into the cytoplasm causes the mobilization of calcium from intracellular stores, whereas diacylglycerol activates protein kinase C (Hill et al., 1997). Stimulation of histamine H_1 receptors has also been shown to stimulate cellular proliferation and to induce the expression of the proto-oncogene c-fos in both human airway (Panettieri et al., 1990) and vascular smooth muscle cells (Satoh et al., 1994). In addition, H_1 receptor stimulation has been shown to induce c-fos expression in hypothalamic neurons (Kjaer et al., 1994) and human T lymphocytes (Kitamura et al., 1996).

The c-fos promoter contains several regulatory sequences in its 5′ untranslated region, which include the serum response element (SRE) and a cAMP response element (CRE) (Hill and Treisman, 1995). At the SRE, a ternary complex forms between serum response factor and a ternary complex factor to mediate responses to growth factors and mitogens via the activation of mitogen-activated protein (MAP) kinases (Hill and Treisman, 1995; Price et al., 1996). MAP kinases are a point of convergence of mitogenic signals from both tyrosine kinase growth factor receptors and G protein-coupled receptors (Hawes et al., 1995; Selbie and Hill, 1998; Robinson and Cobb, 1997). G_i-coupled receptors, such as the α_2-adrenoceptor, have been shown to activate this pathway via a mechanism requiring G protein $\beta\gamma$-subunits, leading to Ras-dependent MAP kinase activation (Hawes et al., 1995; Van Biesen et al., 1996). In contrast, activation of MAP kinase via G_Q-coupled muscarinic M_1 receptors in Cos-7 cells is predominantly via activation of protein kinase C and a Ras-independent pathway (Hawes et al., 1995). However, in CHO cells, the muscarinic M_1 receptor has been reported to couple to pertussis toxin-sensitive G_o proteins and to stimulate MAP kinase via a novel PKC-dependent mitogenic signaling pathway (Van Biesen et al., 1996).

The PKC serine/threonine protein kinase C family is made up of at least 12 different isoforms (Mellor and Parker, 1998). They have been divided into four groups based on amino acid sequence similarity and sensitivity to calcium ions and diacylglycerol (Mellor and Parker, 1998; Almholt et al., 1999).

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ABBREVIATIONS: SRE, serum response element; CRE, cAMP response element; CHO, Chinese hamster ovary; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; PDBu, phorbol 12,13-dibutyrate; PAGE, polyacrylamide gel electrophoresis; AM, acetoxymethyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; IBD, ingenol-3,20-dibenzoate; PMA, phorbol-12-myristate-13-acetate; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase.

The four families are 1) the calcium-dependent conventional PKC isoforms α, βI, βII, and γ; 2) the calcium-independent novel PKC (nPKC) isoforms δ, ϵ, η, θ; 3) the atypical isoforms ι, λ, ζ; and 4) PKCμ and its mouse homolog protein kinase D (Johannes et al., 1994; Newton, 1995; Mellor and Parker, 1998; Almholt et al., 1999). Conventional PKCs and nPKCs are regulated by 1,2-diacylglycerol and phorbol esters, whereas atypical PKCs are not (Johannes et al., 1994; Newton, 1995; Mellor and Parker, 1998; Almholt et al., 1999). Recent studies have begun to provide evidence for the role of specific PKC isoforms in the activation of MAP kinases in different cell types, and in response to particular growth factors (Ueda et al., 1996; Mackenzie et al., 1997; Kim et al., 1999). In the present study, we address the question of whether specific isoforms of PKC mediate the effect of histamine H_1 receptor stimulation on signaling to the c-fos promoter.

Experimental Procedures

Materials. Cell culture flasks and 24-well cluster dishes were from Costar. Dulbecco's modified Eagle's medium/nutrient mix Ham's F-12 medium, L-glutamine, fetal calf serum, forskolin, Hanks' balanced salt solution, HEPES, histamine dihydrochloride, phorbol-12-myristate-13-acetate, phorbol 12,13-dibutyrate, 4α-phorbol, ingenol-3,20-dibenzoate, and mepyramine maleate were supplied by Sigma (Poole, Dorset, UK). The radiochemicals [2,8-^{3}H]adenine, myo-[2-^{3}H]inositol, [^{3}H]mepyramine, and [8-^{14}C]cAMP were obtained from New England Nuclear (Stevenage, Herts, UK). Rolipram, Ro-31-8220, PD 98059, and Gö 6976 were purchased from Calbiochem (Nottingham, UK). All other chemicals were of analytical grade.

Cell Culture. CHO-H1 cells (a clonal CHO-K1 cell line expressing recombinant bovine histamine H_1 receptor at a level of 3 pmol/mg protein; Iredale et al., 1993) were grown at 37°C in a humidified air/CO_2 atmosphere (95:5) in 75-cm^2 flasks. For measurement of c-fos promoter activity, CHO-H1 cells were secondarily transfected with a reporter vector encoding firefly luciferase (pGL3 Basic; Promega, Madison, WI) under the control of the full c-fos promoter (−711 to +1 bases; Shaw et al., 1989), together with a zeocin selectable vector pZeoSV (Invitrogen, San Diego, CA). Cells were subsequently selected with 200 μg/ml zeocin. The cells were grown in Dulbecco's modified Eagle's medium/nutrient mix Ham's F-12 medium (1:1) supplemented with 2 mM L-glutamine and 10% fetal calf serum. Cells for measurement of cAMP accumulation and luciferase activity were grown in 24-well cluster dishes. Cells for Western blot analysis were grown in 100-mm dishes or 162-cm^2 flasks. All experiments were performed on confluent monolayers.

Measurement of Histamine-Stimulated Luciferase Activity. Confluent CHO-H1 cell monolayers, in 24-well cluster dishes, were incubated at 37°C in a humidified air/CO_2 atmosphere (95:5) for 24 h in 1 ml of serum-free DMEM/F-12 media immediately before agonist administration. The medium was aspirated and replaced with 1 ml of fresh serum-free DMEM/F-12 media. Agonists (10 μl) or fetal calf serum (100 μl; total volume 1 ml) was then added and the incubation continued for 6 h. Where appropriate, antagonists were added 30 min before agonist administration. Luciferase activity in cell lysates was then monitored using the Promega luciferase assay system according to the manufacturer's instructions.

Accumulation of [^{3}H]cAMP. Confluent cell monolayers, in 24-well cluster dishes, were incubated for 2 h at 37°C with 0.3 ml of Hanks'/20 mM HEPES buffer, pH 7.4, containing [^{3}H]adenine (74 KBq/well). The cells were washed twice and then incubated for 15 min at 37°C, in 0.3 ml of Hanks'/HEPES buffer containing the cAMP phosphodiesterase inhibitor rolipram at final concentrations of 10 or 100 μM. Signal sizes were identical at both concentrations. When required the protein kinase C inhibitor Ro-31-8220 was added 30 min before agonist administration. Agonists were added in 10 μl of medium and the incubation continued for a further 10 min. Incubations were terminated by the addition of 50 μl of concentrated HCl, and an additional 0.7 ml of Hanks'/HEPES was added to each well. An aliquot (50 μl) of cell lysate was removed to obtain a measure of the amount of [^{3}H]adenine taken up by the cells. [^{3}H]cAMP was then isolated by sequential Dowex-alumina chromatography (Donaldson et al., 1988). To allow for percentage recovery correction, the samples were spiked with [^{14}C]cAMP before being applied to the columns. After elution, levels of [^{3}H]cAMP and [^{14}C]cAMP were determined by liquid scintillation counting.

Cell Extracts for Protein Kinase C Detection after Prolonged Treatment with Phorbol Ester. CHO-H1 cells, grown to 80% confluency in 100-mm petri dishes or T162 flasks, were treated for a further 24 h with the active phorbol ester phorbol 12,13-dibutyrate (PDBu, 1 μM), its inactive analog 4α-phorbol (1 μM), or vehicle control, in Dulbecco's modified Eagle's medium/nutrient mix Ham's F-12 medium (1:1) supplemented with 2 mM L-glutamine, 1% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphoterocin B. In some experiments ingenol-3,20-dibenzoate (3 μM) or phorbol-12-myristate-13-acetate (3 μM) was used in place of PDBu. After this period, the now confluent cell monolayers were washed twice with ice-cold PBS (138 mM NaCl, 2.7 mM KCl, 12.9 mM $Na_2HPO_4{\cdot}2H_2O$, and 1.5 mM KH_2PO_4, pH 7.4) and then harvested from the culture flasks using either a cell scraper or by incubation with 1 mM EDTA in PBS. The detached cells were collected by centrifugation at 700g for 5 min. For experiments looking at the expression of the PKC isoforms, α, δ, and ζ, detergent extracts were prepared from the collected cells by homogenizing them in (100 μl) ice-cold extract buffer [20 mM Tris-HCl, 1% (v/v) Triton X-100, 10 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM leupeptin, 1 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4], for 20 strokes in a hand-held, 1-ml glass homogenizer, and then centrifuging for 15 min at 36,000g, 4°C. The protein content of the supernatant, containing cytosolic and membrane proteins, was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Protein matched samples (15–40 μg) were heated to 95°C in SDS-PAGE sample buffer [0.5 M Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5% 2-mercaptoethanol, 0.05% (v/v) bromphenol blue, pH 6.8], and then subjected to Western blot analysis. Alternatively, for experiments looking at the expression of PKCμ and PKCι, the collected cells were resuspended in 20 mM Tris-HCl, 10 mM EGTA, 10 mM EDTA, 1 mM dithiothreitol, 0.1 mM leupeptin, 1 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4, and the density of total cells estimated in this suspension using a hemocytometer. Volumes of cell suspension containing a fixed number of cells (0.2 million) were directly heated to 95°C in SDS-PAGE sample buffer and then subjected to Western blot analysis.

Membrane Preparation for Protein Kinase C Detection after Acute Stimulation with Histamine and Phorbol Ester. Confluent cell monolayers in 162-cm^2 flasks were washed twice with Hanks'/HEPES (37°C, pH 7.4) and then stimulated for 5 min with either histamine (100 μM), PDBu (1 μM), or vehicle control in Hanks'/HEPES at 37°C. The cells were then immediately transferred to an ice bath, and washed twice with ice-cold PBS. Cells were removed from the flasks using a cell scraper and collected by centrifugation at 1400g for 3 min. The cells from each flask were then resuspended in 500 μl of ice-cold lysis buffer (20 mM Tris-HCl, 10 mM EGTA, 10 mM EDTA, 1 mM dithiothreitol, 0.1 mM leupeptin, 1 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4), and disrupted by either sonication (2 × 5 s, 18 μm, MSE Soniprep 150) or using a Polytron tissue homogenizer (5 s, setting 6; Kinematica GmbH, Lucerne, Switzerland), over ice. The homogenates were centrifuged for 5 min at 200g, to remove any intact cells, and then centrifuged for 15 min at 36,000g, 4°C, to pellet down the cell membranes. The membranes

were resuspended in 50 μl of lysis buffer, and the protein content measured by the method of Bradford (1976), using bovine serum albumin as a standard. Protein matched samples (80–100 μg) were heated to 95°C in SDS-PAGE sample buffer, and then subjected to Western blot analysis.

Western Blot Analysis. Protein samples were separated by SDS-PAGE (7.5% acrylamide gel) using the Bio-Rad Mini-Protean II system. After transfer of proteins to nitrocellulose membranes, the membranes were blocked overnight in blocking buffer [5% (w/v) low-fat dried milk in PBS/0.1% (v/v) Tween 20], at 4°C. The blots were then incubated with primary anti-PKC antibodies (Transduction Laboratories, distributed by Affiniti Research Products Ltd, Exeter, UK) for 2 h at room temperature in blocking buffer. The blots were washed briefly in washing buffer [PBS/0.1% (v/v) Tween 20], then for 15 min, and a further two times for 5 min with fresh changes of the washing buffer. The blots were then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG, Fc-specific, affinity-isolated antibody; Sigma), in blocking buffer, for 1 h at room temperature. The secondary antibody was removed, the blots washed twice briefly with washing buffer, then for 15 min and a further four times for 5 min, before developing the blots using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). For the studies looking at the prolonged effect of PDBu treatment on PKCα expression, an alternative primary anti-PKC antibody was used (Life Technologies, Gaithersburg, MD). The washing buffer used was PBS/1% (v/v) Tween 20, the blocking buffer additionally contained 1% BSA (w/v), and the secondary antibody was horseradish peroxidase-conjugated swine anti-rabbit IgG supplied by DAKO (Glostrup, Denmark). Otherwise, all the other conditions of Western blot analysis were identical.

Phosphatidylinositide Turnover. Confluent monolayer cultures were loaded for 24 h with myo-[^{3}H]inositol (37 kBq/well) in 24-well cluster dishes in inositol-free DMEM containing 2 mM glutamine, 1% fetal calf serum, and PDBu or 4α-phorbol as required. After being washed twice with 1 ml/well of Hanks'/HEPES buffer, pH 7.4, the cells were incubated for 30 min at 37°C in 290 μl/well of Hanks'/HEPES buffer containing 20 mM LiCl and Ro-31-8220 or PDBu as required. Agonists were added in 10 μl of medium and the incubation continued for 40 min. Incubations were terminated by aspiration of the incubation medium and addition of 900 μl of cold (−20°C) methanol/0.12 M HCl (1:1, v/v). The cells were left at −20°C for at least 2 h before isolating total [^{3}H]inositol phosphates as described previously (White et al., 1992). Total [^{3}H]inositol phosphate levels were determined by liquid scintillation counting.

[^{3}H]Mepyramine Binding to Intact CHO-H1 Cells. Binding of [^{3}H]mepyramine to cell surface H_1 receptors was measured by a modification of the method of Hishinuma and Young (1995). Confluent monolayers, in 48-well cluster dishes, were incubated with [^{3}H]mepyramine (saturation binding assays 0.5–16 nM; displacement assays 2 nM) in the absence or presence of the quaternary H_1 antagonist pirdonium (10 μM), in Hanks'/HEPES, pH 7.4, for 1 h at 37°C. This incubation was terminated by aspirating off the media to waste, washing the wells with Hanks'/HEPES, and then detaching the cells with 5% (w/v) trypsin/2% EDTA in 0.9% NaCl. The amount of [^{3}H]mepyramine bound to the detached cells was determined by liquid scintillation spectrometry, using the scintillant, Emulsifier Scintillator Plus (Canberra Packard Canada, Mississauga, ON, Canada).

Ca^{2+} Measurements. Changes in intracellular Ca^{2+} concentrations were measured essentially as described previously (Iredale et al., 1993). Briefly, the monolayers from near confluent 75-cm^2 flasks (one flask per three time courses) were detached with trypsin/EDTA (0.5 g of trypsin, 0.2 g of EDTA, and 0.85 g/l NaCl) and resuspended in a simple saline buffer (2 mM $CaCl_2$, 145 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, and 10 mM HEPES, pH 7.45). The resuspended cells were then incubated with the fluorescent Ca^{2+} indicator fura-2 acetoxymethyl ester (fura-2 AM; 5 μM), in the presence of 10% fetal calf serum, for 20 min at 37°C. When required the Ca^{2+} chelator BAPTA/AM (50 μM) was also included during this 20-min incubation. The incubation was continued for a further 5 min, after a 3-fold dilution, to ensure maximum hydrolysis of ester to the acid form. At the end of this loading period, excess dye was removed by centrifugation, and the cells were resuspended in fresh buffer (no serum) and left at room temperature until use. Each Ca^{2+} time course was preceded by a rapid spin in a microcentrifuge followed by resuspension in 2 ml of buffer containing 0.1 mM EGTA and no added $CaCl_2$.

All experiments were carried out using a PerkinElmer LS50B spectrometer (PerkinElmer, Norwalk, CT), with excitation ratios between 340 and 380 nm, recording at 500 nm. The time course for each Ca^{2+} measurement was 100 s with drugs added in 20-μl volumes. At the end of a time course, either $CaCl_2$ (20 mM) followed by ionomycin (20 μM) was added to determine R_{max}, ionomycin (20 μM) followed by EGTA (6.25 mM, pH > 8.5) to determine R_{min}, or $MnCl_2$ (5 mM) followed by ionomycin (20 μM) to determine autofluorescence. (In some experiments autofluorescence was measured, alternatively, by simply measuring the fluorescence produced by an equivalent suspension of cells not loaded with fura-2 AM). Using these values and those obtained with fura-2 free acid, intracellular Ca^{2+}concentrations were calculated according to the method of Grynkiewicz et al. (1985).

Data Analysis. Agonist concentration-response curves were fitted to a logistic equation using the nonlinear regression program Prism (GraphPad Software, San Diego, CA). The equation fitted was: Response = $(E_{max} \times A^{n_H}) / [(EC_{50})^{n_H} + A^{n_H}]$, where E_{max} is the maximal agonist response, A is the agonist concentration, and n_H is the Hill coefficient.

Results

Histamine H_1 Receptor-Stimulated Gene Expression. CHO-H1 cells, expressing a luciferase reporter gene (pGL3 Basic) under the transcriptional control of the human c-fos promoter, responded to a variety of different stimuli, including 10% fetal calf serum, histamine, thrombin, the adenylyl cyclase activator forskolin, and the phorbol ester PDBu (Fig. 1). The response to histamine (EC_{50} = 9.8 ± 0.9 nM; n = 14) was antagonized by the H_1 receptor-selective antagonist mepyramine (100 nM; apparent K_D = 0.65 ± 0.19

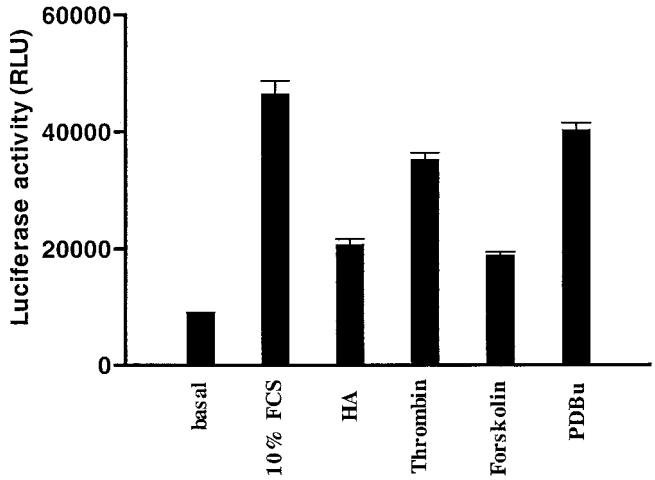

Fig. 1. Influence of different mitogens on human c-fos promoter-regulated luciferase expression in CHO-K1 cells expressing the bovine histamine H_1 receptor. After 24 h in serum-free medium, agonist incubation was for 6 h. Histamine (HA; 100 μM), thrombin (1 U/ml), forskolin (3 μM), and PDBu (1 μM). FCS, fetal calf serum. Data represent mean ± S.E.M. of triplicate determinations in a representative experiment. Luciferase activity is represented as relative light units (RLU). Similar data were obtained in two further experiments.

nM; $n = 3$), although there was a small reduction in the maximal response to histamine (Fig. 2). Luciferase responses of similar magnitude were also obtained with a range of selective H_1 agonists (Table 1). The luciferase response to histamine was not significantly modified by pretreatment of cells with pertussis toxin (24 h; 100 ng/ml), although approximately 50% of the response to 10% fetal calf serum (presumably that component mediated by lysophosphatidic acid) was attenuated by this treatment (Fig. 3).

Role of Protein Kinase Cα in Histamine H_1 Receptor-Mediated Gene Expression. The bisindolylmaleimide PKC inhibitor Ro-31-8220 (10 μM), completely inhibited the c-fos promoter-mediated activation of luciferase expression induced by histamine (Fig. 4a). However, in marked contrast, Ro-31-8220 (10 μM) produced a small leftward shift in the concentration-response curve for histamine obtained from activation of [^{3}H]inositol phosphate accumulation (Fig. 5). These latter data confirm that Ro-31-8220 does not act as an inhibitor at the level of the histamine H_1 receptor. The c-fos-luciferase response to histamine was also completely attenuated after pretreatment of CHO-H1 cells with the protein kinase C activator phorbol-12-myristate-13-acetate (3 μM) for 24 h (Fig. 4b). Such treatment also completely attenuated the activation of the c-fos promoter by PDBu (1 μM). Interestingly, both Ro-31-8220 (10 μM) and 24 h PDBu treatment (1 μM) reversed the acute inhibitory effects of PDBu (1 μM; 30 min) on the [^{3}H]inositol phosphate response to 300 nM histamine (Table 2), which are presumably due to phosphorylation of histamine H_1 receptor protein (Fujimoto et al., 1999). Pretreatment (24 h) of CHO-H1 cells with PDBu (1 μM) did not alter the parameters of saturable [^{3}H]mepyramine binding in intact cells compared with the parameters after treatment with 4α-phorbol (4α-phorbol, 1 μM: B_{max} = 136 ± 7 fmol/well, pK_D = 8.78 ± 0.05; PDBu: B_{max} = 132 ± 12 fmol/well, pK_D = 8.89 ± 0.11; $n = 3$).

Several isoforms of protein kinase C could be detected in cell extracts from CHO-H1 cells by Western blot analysis (Fig. 6a). The apparent molecular weights of the isoforms were 84 ± 1 (α; $n = 17$), 80 ± 1 (δ; $n = 16$), 117 ± 3 (μ; $n = 21$), 79 ± 1 (ι; $n = 11$) and 75 ± 1 (ζ; $n = 10$) in close agreement with values reported in the literature (Selbie et al., 1993; Johannes et al., 1994; Mackenzie et al., 1997). No signal for PKCθ was found, and only small and not very reproducible signals to PKCβ and PKCϵ (Fig. 7c). The effect of prolonged phorbol ester treatment on those protein kinase C isoforms that are readily detected by Western blot analysis

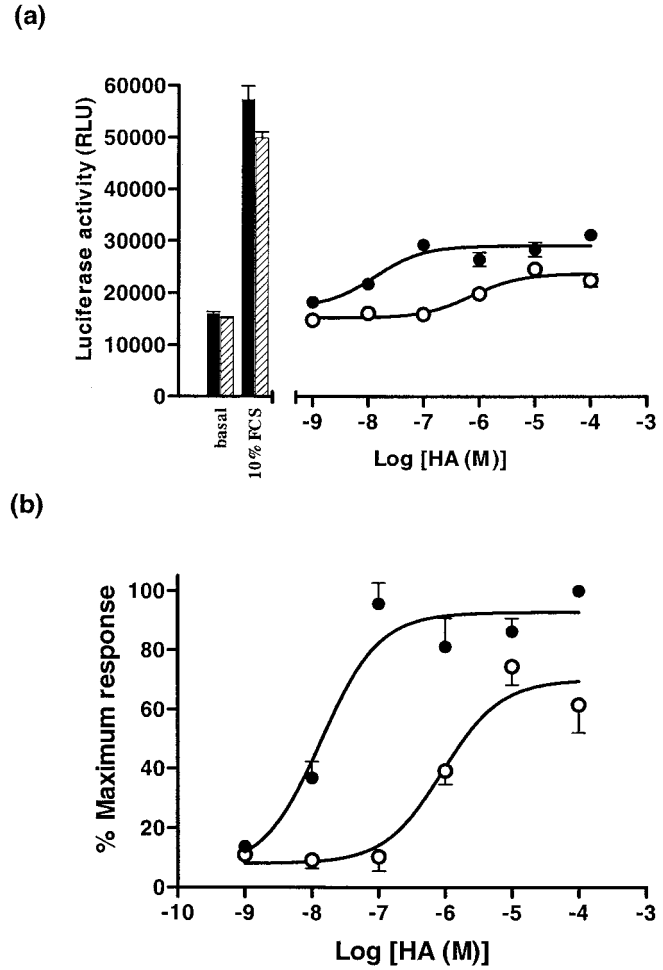

Fig. 2. Antagonism by the selective H_1 receptor antagonist mepyramine of histamine-stimulated c-fos-luciferase expression in CHO-H1 cells. Cells were serum-starved for 24 h before the addition of agonists as described under *Experimental Procedures*. Cells were incubated for 30 min in the presence or absence of 100 nM mepyramine before addition of histamine in 10 μl of medium. a, representative experiment (●, control; ○, +mepyramine; ■, control; ▨, +mepyramine). Values represent mean ± S.E.M. of triplicate determinations. b, combined data from three experiments (●, control; ○, +mepyramine). Data (mean ± S.E.M.) are expressed as a percentage of the control response to 100 μM histamine, measured in each experiment.

TABLE 1

Concentration-response parameters for histamine H_1 agonist-stimulated c-fos-luciferase expression.

Values represent mean ± S.E.M. Number of experiments given in parentheses.

Agonist	Log EC_{50}	Maximal Response (Fold over Basal)	Relative Potency
	M		
Histamine	−8.03 ± 0.04 (14)	1.86 ± 0.07 (18)	100
N^{α}-Methylhistamine	−7.45 ± 0.13 (4)	2.13 ± 0.18 (4)	26.3
2-Thiazolylethylamine	−7.03 ± 0.10 (4)	1.90 ± 0.08 (4)	10.0
2-Methylhistamine	−6.69 ± 0.12 (4)	1.78 ± 0.08 (4)	4.6
2-Pyridylethylamine	−6.14 ± 0.09 (4)	1.78 ± 0.09 (4)	1.3

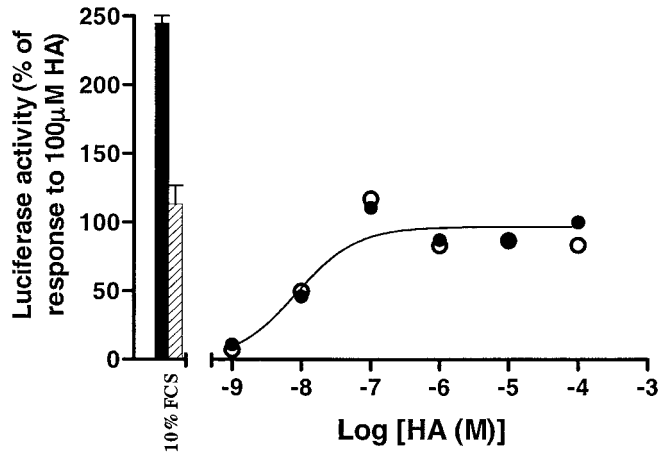

Fig. 3. Effect of pertussis toxin on histamine-stimulated c-fos-luciferase expression. Cells were incubated in serum-free medium and, where appropriate, pertussis toxin (PTX, 100 ng/ml) for 24 h before histamine administration (●, control; ○, +PTX; ■, control; ▨, +PTX). Histamine (HA) or 10% fetal calf serum (FCS) was added for 6 h. Values represent mean ± S.E.M. of triplicate determinations in three separate experiments. Data are expressed as a percentage of the control response to 100 μM histamine, which was measured in each individual experiment.

is shown in Fig. 6. The expression of the phorbol ester-sensitive isoforms PKCα and PKCδ, but not the atypical isoforms PKCι, PKCμ, and PKCζ, were down-regulated by this treatment (Fig. 6a). Of the two isoforms that were down-regulated, only protein kinase Cα was translocated to CHO-H1 cell membranes after stimulation with histamine (Fig. 6b).

The diterpene ingenol-3,20-dibenzoate (IBD) has been suggested to be a selective activator of novel PKC isoforms (δ, ϵ, and θ) and PKCμ (Asada et al., 1998). In the present study, pretreatment with IBD (3 μM; 24 h) was used to investigate the potential role of PKCδ in the c-fos-luciferase response to histamine. Under these conditions, IBD (24 h) completely attenuated the responses to both histamine and PDBu (Fig. 7, a and b). Interestingly, the response to IBD (1 μM) was also completely attenuated by 24-h treatment with PMA (3 μM; Fig. 7a). However, Western blot analysis revealed that IBD (3 μM; 24 h) treatment was able to down-regulate PKCα in addition to PKCδ (Fig. 7c). As an alternative approach, the cPKC and PKCμ-selective inhibitor Gö 6976, which has negligible effect on nPKCs, was used (Martiny-Baron et al., 1993; Gschwendt et al., 1996; Way et al., 2000). Gö 6976 was able to markedly attenuate the activation of the c-fos promoter by histamine in CHO-H1 cells, consistent with a role for PKCα (Fig. 8).

Role of Intracellular Calcium. We have previously shown that H_1 receptor stimulation in populations of CHO-H1 cells causes a robust and biphasic increase in intracellular Ca^{2+} levels consistent with release from intracellular stores and subsequent capacititative reentry (Iredale et al., 1993). In the absence of extracellular calcium, the c-fos-luciferase response to histamine was significantly attenuated (Fig. 9a). Under these conditions, however, histamine was

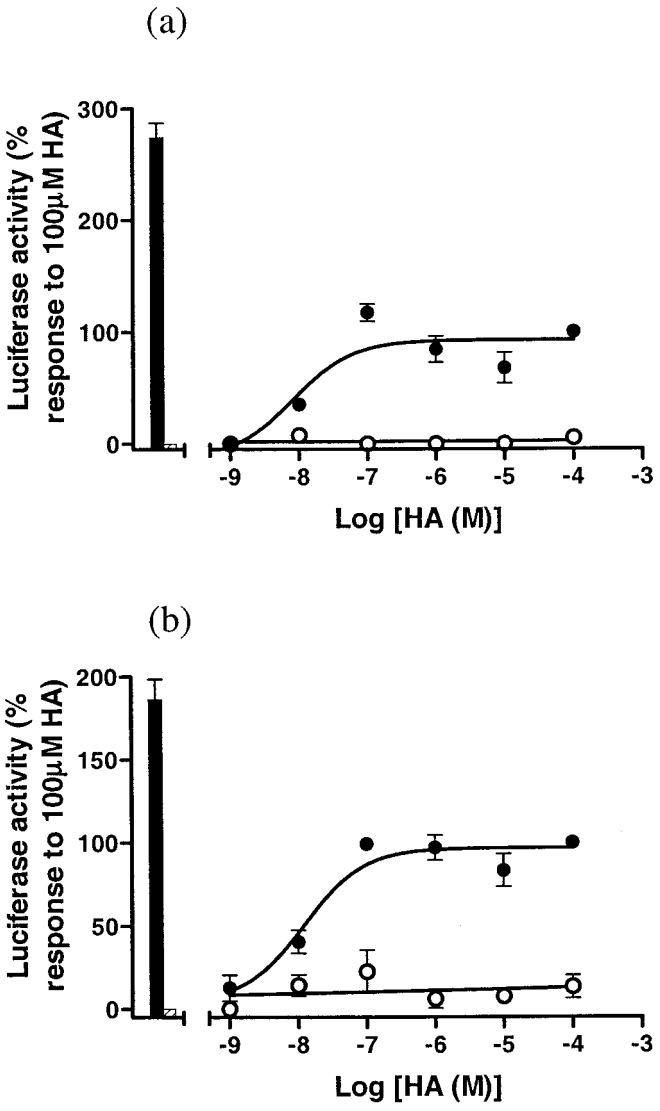

Fig. 4. Influence of the PKC inhibitor Ro-31-8220 (a) and 24-h pretreatment with PMA on histamine-stimulated c-fos-luciferase expression (b). a, cells were serum-starved for 24 h, media was replaced with fresh serum-free media containing 10 μM Ro-31-8220 or vehicle control, and incubated at 37°C in an atmosphere of 5% CO_2 in air for 30 min before agonist addition (in 10 μl of medium) for a further 5 h (●, control; ○, +Ro-31-8220; ■, control; ▨, +Ro-31-8220). b, cells were incubated in serum-free medium containing PMA (3 μM) or vehicle control for 24 h. The medium was then replaced with fresh medium, agonist was then added in 10 μl of medium, and the incubation continued for 5 h (●, control; ○, +PMA; ■, control; ▨, +PMA). Data are expressed as a percentage of the control response to 100 μM histamine. The histograms show the response to PDBu (1 μM) under the two conditions. Values represent mean ± S.E.M. of triplicate determination in three (a) or four (b) separate experiments.

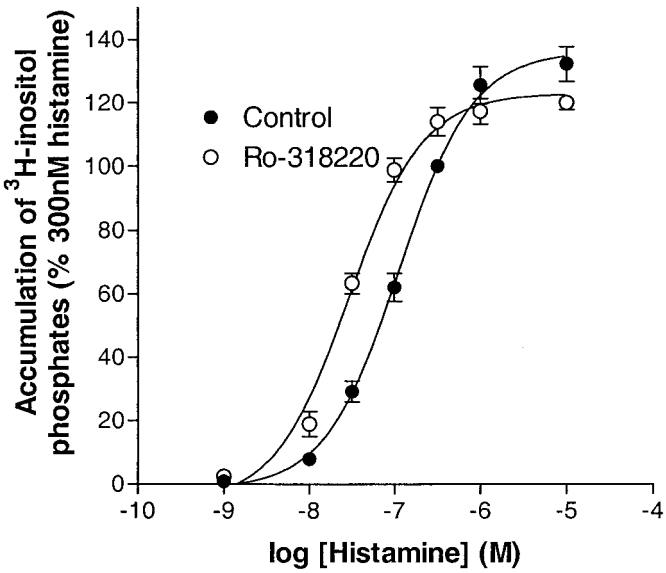

Fig. 5. Effect of Ro-31-8220 (10 μM) on histamine-stimulated [^{3}H]inositol phosphate accumulation in CHO-H1 cells. Values represent mean ± S.E.M. of triplicate determinations in three separate experiments. In each experiment data have been expressed as a percentage of the response to 0.3 μM histamine measured under control conditions.

TABLE 2

Inhibition of histamine-stimulated total [^{3}H]inositol phosphate accumulation in CHO-H1 cells by PDBu

The inhibition was reversed by cotreatment with 10 μM Ro-31-8220 or after 24-h preincubation with 1 μM PDBu. The accumulation of total [^{3}H]inositol phosphates was measured as described under *Experimental Procedures.* Values represent mean ± S.E.M. from three experiments. Data are expressed as a percentage of the control response to 300 nM histamine alone.

	% Histamine Response		
	Control	Ro-31-8220 (10 μM)	24 h PDBu (1 μM)
Control (4α-phorbol)	105 ± 3	118 ± 2	118 ± 8
PDBu	60 ± 3*	120 ± 6	120 ± 4

* Response after 30-min PDBu (1 μM) pretreatment was significantly lower ($P < 0.01$) than the control response obtained after parallel pretreatment with 4α-phorbol (1 μM).

able to produce a substantial increase in intracellular free calcium ion concentration that could be abolished by incubation with the cell-permeant calcium chelator BAPTA/AM (Fig. 9c). Pretreatment of cells with BAPTA/AM (50 μM) markedly inhibited both histamine-stimulated and basal luciferase expression (Fig. 9a). Histamine produced an increase in intracellular calcium over the same concentration range as that required to stimulate inositol phosphate accumulation (compare Figs. 9d and 5). Ionomycin (1μM) (which produced the same size calcium response as maximally effective concentrations of histamine; Fig. 9d), however, was unable to stimulate the c-fos promoter (Fig. 9b).

Role of MEK-1 and cAMP. To establish whether the response to histamine is mediated via the MAP kinase pathway we have used the cell-permeant MEK-1 inhibitor PD 98059 (50 μM; Waters et al., 1995). This compound was able to markedly attenuate (although not completely attenuate) the stimulation of c-fos-regulated luciferase activity by histamine (Fig. 10), confirming that the MAP kinase pathway is the likely major target for PKCα actions at the level of Raf-1 (Hawes et al., 1995). However, it is notable in Fig. 1 that the adenylyl cyclase activator forskolin can also activate transcription via the CRE site within the c-fos promoter. In these cells, histamine can elicit a stimulation of cAMP accumulation of similar magnitude to 1 μM forskolin (Fig. 11a). It is therefore possible that histamine is able to produce some of its effect via cAMP production, phosphorylation of cAMP response element-binding protein and stimulation of the CRE in the c-fos promoter. Pretreatment (24 h) with PDBu (1 μM), however, did not alter the ability of histamine to stim-

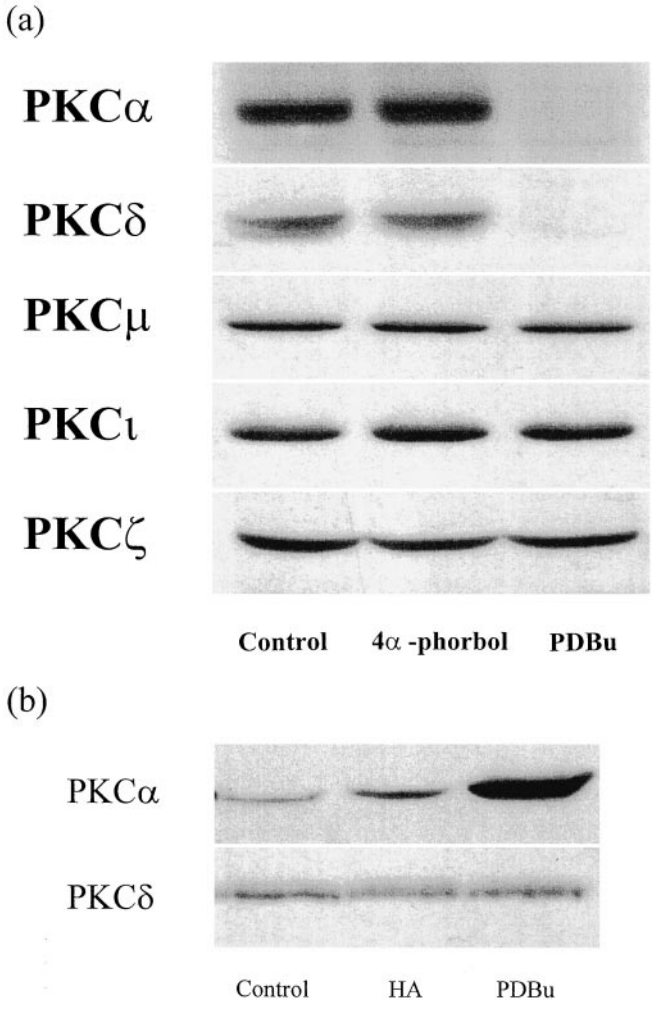

Fig. 6. PKC isoforms in CHO-H1 cells. a, down-regulation of PKC isoforms by pretreatment with PDBu. Cells were preincubated for 24 h with 1 μM PDBu, an inactive analog 4α-phorbol (1 μM) or vehicle (dimethyl sulfoxide) as a control. b, translocation of PKC isoforms to CHO-H1 cell membranes after stimulation (5 min) of intact cell monolayers with histamine (HA, 100 μM) or PDBu (1 μM). Cell extracts were prepared and analyzed for the expression of PKC isoforms by Western blot analysis as described under *Experimental Procedures*. Data are from a single experiment and are representative of at least two other experiments.

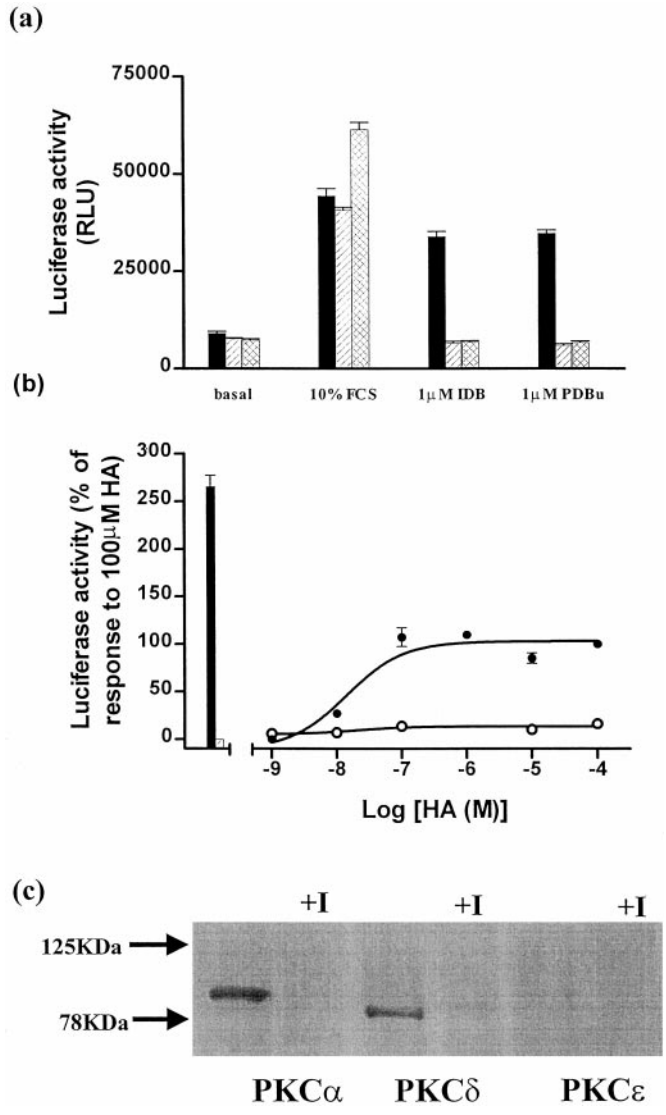

Fig. 7. Influence of IDB on PKC isoforms and agonist-stimulated c-fos-luciferase expression in CHO-H1 cells. a, effect of 24-h pretreatment with either IDB (3 μM) or PMA (3 μM) on luciferase responses to 10% fetal calf serum (FCS), 1 μM IDB or 1 μM PDBu (■, control; ▨, +IDB, 24 h; ▩, +PMA, 24 h). Agonist incubation was for 5 h. Values represent mean ± S.E.M. of triplicate determinations. Similar data were obtained in two further experiments. b, effect of 24-h pretreatment with IDB (3 μM) on responses to histamine (HA). The histograms show the responses to IDB (1 μM) in control cells and cells pretreated with IDB (3 μM; 24 h) (●, control; ○, +IDB, 24 h; ■, IDB; ▨, +IDB, 24 h). Values represent mean ± S.E.M. of triplicate determinations in three separate experiments. Data are expressed as a percentage of the response to 100 μM histamine, which was measured in each individual experiment. c, down-regulation of PKCα, δ, and ϵ by pretreatment with IDB (3 μM; 24 h). PKCμ, ι, ζ, and β were also present but not down-regulated.

ulate cAMP accumulation in these cells (Fig. 11b). These data suggest that the influence of histamine on cAMP accumulation is not mediated via PKCα.

Discussion

Previous studies have shown that distinct pathways can mediate the effect of G_i- and G_Q-coupled receptors on MAP kinase activation (Hawes et al., 1995). Thus, although G_i-coupled receptors stimulate the MAP kinase pathway via $G_{\beta\gamma}$-subunits and Ras, G_Q-coupled receptors stimulate MAP kinase activation and cell proliferation via PKC and c-Raf (Hawes et al., 1995). However, in certain cells (e.g., PC12 cells) activation of protein kinase C is not required for activation of MAP kinase pathways via G_Q-coupled receptors such as the α_{1A}-adrenoceptor (Berts et al., 1999). Furthermore, evidence has accrued that some G_Q-coupled receptors, notably the muscarinic M_1 receptor in CHO cells (Van Biesen et al., 1996) and the α_1-adrenoceptor in oligodendrocyte progenitor cells (Khorchid et al., 1999), can also stimulate MAP kinase activation and c-fos expression via pertussis toxin-sensitive G proteins and diacylglycerol-dependent PKC isoforms.

In the present study we have provided evidence that the stimulation of c-fos promoter activity by histamine H_1 receptor activation in CHO-K1 cells is not mediated by a pertussis toxin-sensitive G protein. The c-fos response to histamine is, however, clearly mediated by a 1,2-diacylglycerol-sensitive PKC isoform because it can be completely attenuated by 24-h pretreatment with a number of different phorbol esters. Under the same conditions, treatment with PDBu (24 h) did not alter the expression of cell surface H_1 receptors in these cells. This was assessed using the H_1-selective radioligand

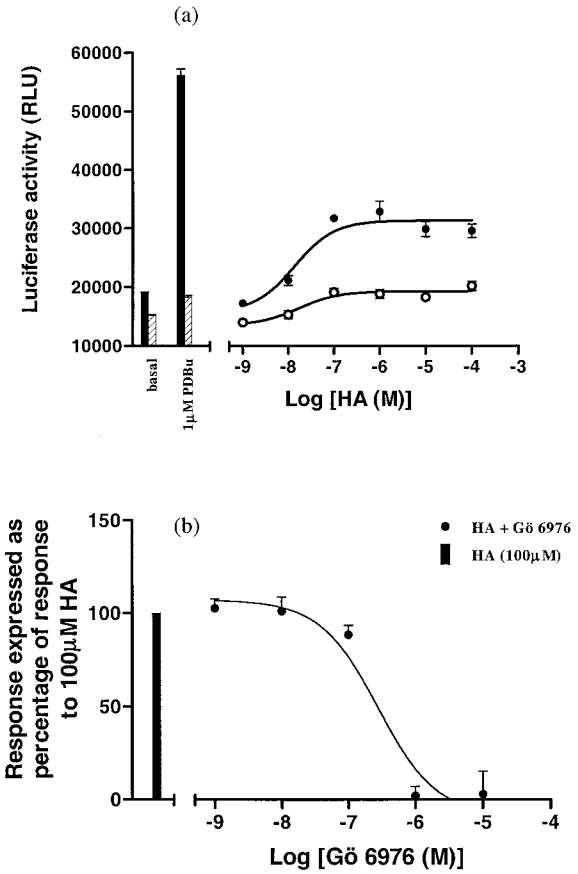

Fig. 8. Influence of the PKC inhibitor Gö 6976 on histamine-stimulated c-fos-luciferase expression. a, effect of 1 μM Gö 6976 on concentration-response curves to histamine [●, control; ○, +Gö 6976 (1 μM); ■, control; ▨, +Gö 6976 (1 μM)]. Agonist incubation was for 5 h. Gö 6976 was added 30 min before histamine. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained on three further occasions. b, concentration-dependent inhibition of the response to 100 μM histamine. Values represent mean ± S.E.M. of triplicate determinations in three separate experiments. Data are expressed as a percentage of the control response to histamine (100 μM) alone.

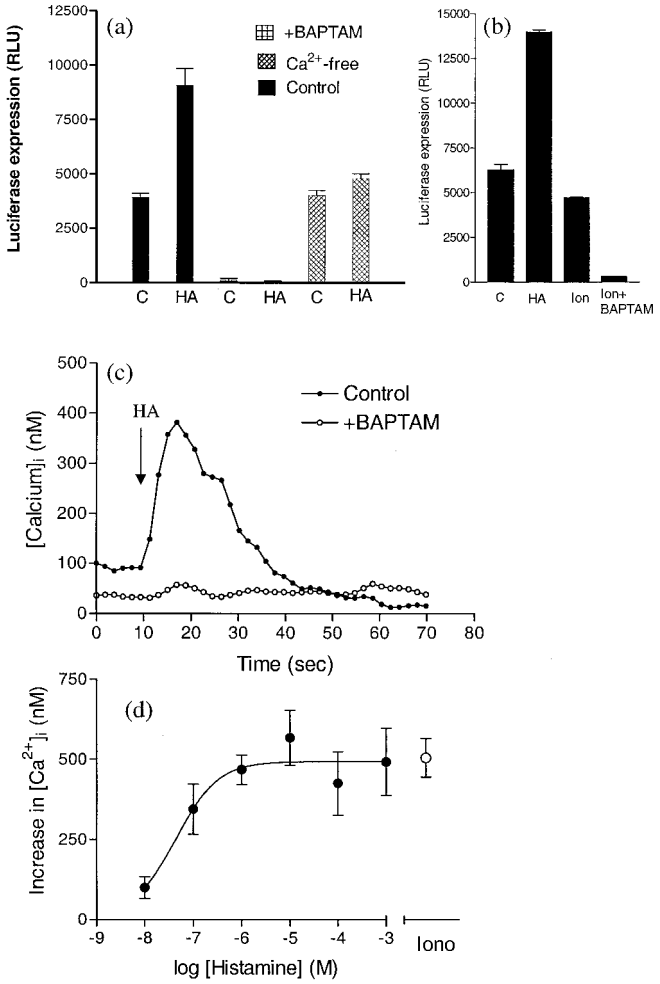

Fig. 9. Role of calcium ions in the stimulation by histamine of c-fos-luciferase expression. a, comparison of responses to histamine (100 μM) in calcium-containing media, calcium-free media, and media containing 50 μM BAPTA/AM. Histograms marked C show the basal responses under each treatment condition. Data represent mean ± S.E.M. from triplicate determinations. Similar data were obtained in three other experiments. b, influence of ionomycin (Ion; 1 μM) on c-fos-luciferase expression in the presence and absence of 50 μM BAPTA/AM. Data represent mean ± S.E.M. from triplicate determinations. Similar data were obtained in three other experiments. c, effects of BAPTA/AM on histamine (HA; 100 μM) stimulated Ca^{2+} transients in CHO-H1 cells assayed in media containing 0.1 mM EGTA and no added Ca^{2+}. Responses from control cells and those preloaded with BAPTA/AM (50 μM) are shown using closed and open symbols, respectively. Each of the time courses shown is representative of at least two others. d, concentration-response curve for histamine-stimulated Ca^{2+} mobilization in CHO-H1 cells. Data were obtained in calcium-free media containing 0.1 mM EGTA. The calcium response to ionomycin (Iono; 1 μM) obtained under identical conditions is also shown for comparison. Data represent the mean ± S.E.M. of six (histamine) or seven (ionomycin) separate experiments.

[^{3}H]mepyramine and the quaternary H_1 antagonist pirdonium to define cell surface binding (Hishinuma and Young, 1995). However, short-term exposure to PDBu (30 min) did inhibit by about 40% the ability of histamine to stimulate phospholipase C, suggesting that 1,2-diacylglycerol and PKC activation can mediate a negative feedback at the level of the H_1 receptor. Indeed, phosphorylation sites for PKC on the histamine H_1 receptor protein have been recently described (Fujimoto et al., 1999). This desensitization effect of PKC was prevented by 24-h PDBu treatment and by the PKC inhibitor Ro-31-8220, and revealed a small enhancement of histamine H_1 receptor activity consistent with a role for diacylglycerol-sensitive PKC isoforms in a negative feedback loop after activation of phospholipase C (Table 2). Consistent with this hypothesis is the fact that Ro-31-8220 was able to produce a small potentiation (i.e., left-shift in the concentration-response curve) of the histamine-stimulated [^{3}H]inositol phosphate accumulation (Fig. 5).

The nonselective inhibitor of PKC isoforms Ro-31-8220 was also able to inhibit completely the c-fos response to histamine, confirming a role for PKC isoforms in this latter response. Treatment (24 h) with PDBu or PMA produced a down-regulation of PKCα, PKCδ, and PKCϵ (the latter being only detectable at low levels under control conditions), but not the atypical isoforms PKCι, PKCμ, and PKCζ, which were also identified in these cells. The c-fos-luciferase response to histamine was markedly inhibited by the MEK-1 inihibitor PD 98059 consistent with an involvement of the Raf-MEK-MAP kinase pathway in signaling to the c-fos promoter. A number of previous studies with G_Q-coupled receptors have indicated that the calcium-independent isoforms PKCδ and PKCϵ are involved in the activation of MAP kinase (Ueda et al., 1996; Mackenzie et al., 1997; Soltoff et al., 1998; Kim et al., 1999). Thus, growth hormone receptors (in 3T3-F44A cells) and P_{2Y2} purinoceptors (in PC12 cells) stimulate MAP kinase pathways via PKCδ (Mackenzie et al., 1997; Soltoff et al., 1998), whereas muscarinic M_3 receptors use PKCϵ (Kim et al., 1999).

Stimulation of H_1 receptors in these cells, or addition of PDBu, produced a translocation of PKCα to CHO-H1 cell membranes but had no effect on the distribution of PKCδ (Fig. 6b). These data suggest that PKCα is primarily responsible for both the short-term inhibitory effect of phorbol esters on histamine-stimulated [^{3}H]inositol phosphate accumulation and the longer term action of histamine and phorbol esters on c-fos-promoter-regulated luciferase expression. In an attempt to selectively activate and down-regulate PKCδ and PKCϵ, we have used the diterpene IBD, which has been reported to be a selective activator of novel PKC isoforms (Asada et al., 1998). Unfortunately, 24-h treatment with this compound produced a complete down-regulation of PKCα in addition to both PKCδ and PKCϵ. However, the indolocarbazole PKC inhibitor Gö 6976, which inhibits cPKC isoforms and PKCμ but not nPKCs (Gschwendt et al., 1996; Martiny-Baron et al., 1993; Way et al., 2000) was able to markedly attenuate the activation of the c-fos promoter by histamine.

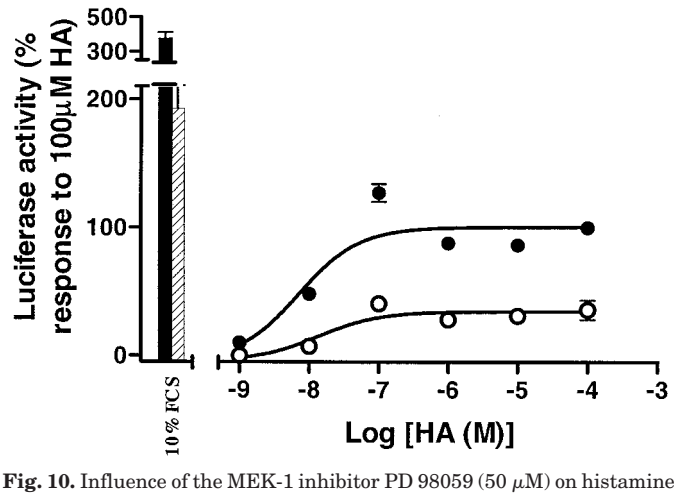

Fig. 10. Influence of the MEK-1 inhibitor PD 98059 (50 μM) on histamine (HA)-stimulated luciferase expression (●, control; ○, +PD98059; ■, control; ▨, +PD98059). PD 98059 was added 30 min before histamine. Agonist stimulation was for 5 h. Values represent mean ± S.E.M. of triplicate determinations in four separate experiments. Data are expressed as a percentage of the control response to 100 μM histamine, which was measured in each experiment.

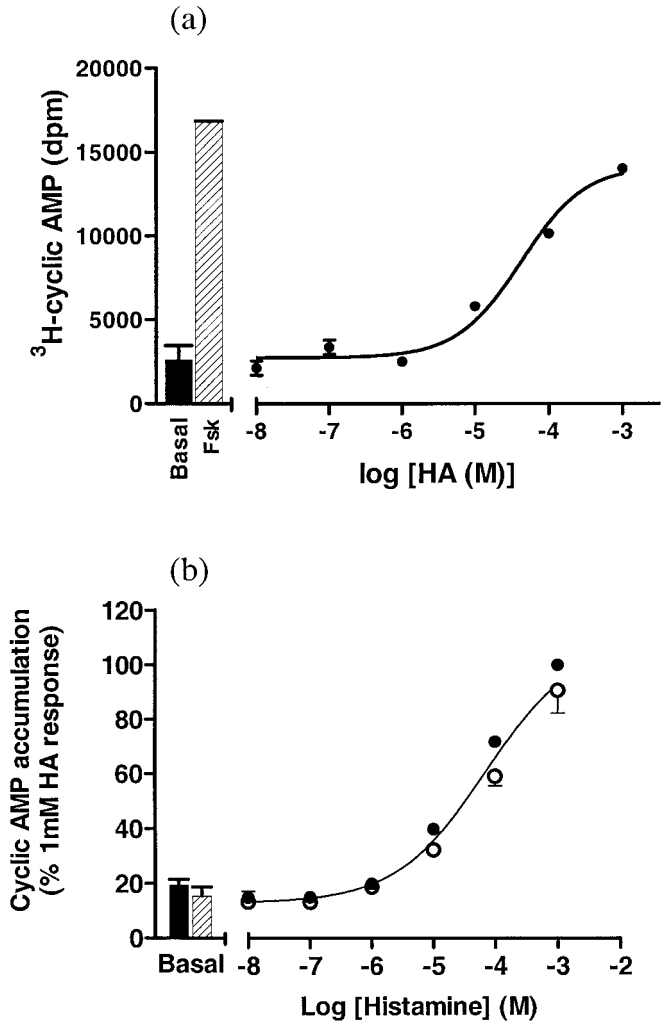

Fig. 11. Effect of histamine on cAMP accumulation in CHO-H1 cells. a, concentration-dependent stimulation by histamine of [^{3}H]cAMP accumulation. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in two further experiments. Histograms show the basal [^{3}H]cAMP levels and the response to 1 μM forskolin (Fsk). b, effect of prolonged PDBu treatment on histamine-stimulated [^{3}H]cAMP accumulation in CHO-H1 cells. Responses from control cells (24-h treatment with 4α-phorbol; 1 μM) are shown by closed symbols and those after preincubation with PDBu (24 h; 1 μM) by open symbols. Data are mean ± S.E.M. of triplicate determinations in three experiments.

At 1 μM, Gö 6976 produced a maximal inhibition of the histamine response (Fig. 8b), although there was still a small residual response to both histamine and PDBu (Fig. 8a). These data suggest that PKCα plays a major role in signaling to the nucleus from the histamine H_1 receptor.

Previous studies in Cos-7 cells have shown that dominant negative PKCα can inhibit the stimulation of the MAP kinase pathway by phorbol esters (Schonwasser et al., 1998). Furthermore, transient transfection of a constitutively active mutant version of PKCα into Cos-7 cells was able to activate MAP kinase, MEK-1, and c-Raf-1 (the kinase that phosphorylates MEK-1) (Schonwasser et al., 1998). In this latter case, the activation of Raf-1 by PKCα seems to be by direct phosphorylation (Sozeri et al., 1992; Kolch et al., 1993). The data presented here are therefore consistent with the sequential activation of a pathway involving PKCα, c-Raf-1, MEK-1, and MAP kinase after activation of phospholipase C by H_1 receptor stimulation. It is also very likely that PKCα mediates a negative feedback at the level of the H_1 receptor because 24-h treatment with PDBu is able to attenuate the inhibitory effects of PKC activation on this response.

The involvement of a calcium-sensitive PKC isoform such as PKCα is also consistent with the data obtained under low intracellular calcium concentrations (Fig. 9). Histamine was able to produce a marked stimulation of intracellular calcium levels from circa 100 nM to a maximal response of 400 to 600 nM (Fig. 9, c and d). In the absence of extracellular calcium ions, there was a substantial attenuation of the c-fos-luciferase response under conditions in which a large but transient change in intracellular calcium could still be observed (Fig. 9, a and c). In the presence of 50 μM BAPTA/AM (which markedly reduced basal calcium levels and prevented the transient histamine response), the luciferase response to histamine was also abolished. Interestingly, there was also a major reduction in the basal level of c-fos-promoter-regulated luciferase expression.

The c-fos promoter contains several regulatory sequences in its 5′-untranslated region, which include the SRE and a CRE (Hill and Treisman, 1995). At the SRE, a ternary complex forms between serum response factor and a ternary complex factor to mediate responses to growth factors and mitogens via the activation of MAP kinases (Hill and Treisman, 1995; Price et al., 1996). MAP kinases are a point of convergence of mitogenic signals from both tyrosine kinase growth factor receptors and G protein-coupled receptors (Hawes et al., 1995; Robinson and Cobb, 1997; Selbie and Hill, 1998).

It is most likely that the pathway involving PKCα used by the histamine H_1 receptor in stimulating the c-fos promoter acts at the level of the SRE via activation of MAP kinases. It is possible that changes in intracellular levels of both calcium and cAMP may also contribute to activated gene transcription at the level of the CRE (Cruzalegui and Bading, 2000). However, mobilization of intracellular calcium by low concentrations of ionomycin (which produced the same calcium response to that obtained with histamine) did not elicit a stimulation of c-fos promoter activity. The adenylyl cyclase activator forskolin did produce a significant activation of luciferase expression. Furthermore, histamine was able to produce a marked change in cAMP accumulation in CHO-H1 cells. However, these changes in cAMP levels did not seem to be secondary to PKCα activation because this effect was unaltered by 24-h treatment with phorbol esters (Fig. 11). It is also notable that the concentrations of histamine necessary to activate maximal c-fos-promoter activity are over 3 orders of magnitude lower than those required to stimulate cAMP accumulation.

In summary, these studies have shown that PKCα plays a major role in the ability of the histamine H_1 receptor to signal to the nucleus. The data obtained are consistent with a pathway involving PKCα, MEK-1, and MAP kinase leading to stimulation of the c-fos promoter SRE, after activation of phospholipase C by H_1 receptor stimulation. Histamine H_1 receptors have classically been associated with the early responses (e.g., smooth muscle contraction, increased capillary permeability) of immediate hypersensitivity reactions. However, there is now accumulating evidence that H_1 receptor activation may also have an important role in the late phase of asthmatic reactions (Roquet et al., 1997). The results obtained in the present manuscript, coupled with the fact that histamine H_1 receptors can induce the immediate early gene c-fos in human airway smooth muscle and inflammatory cells (Panettieri et al., 1990; Kitamura et al., 1996), suggests that signaling to the nucleus via PKCα after H_1 receptor activation may have important physiological and pathophysiological consequences.

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